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HLA-Cw*03-Restricted CD8$^+$ T-Cell Responses Targeting the HIV-1 Gag Major Homology Region Drive Virus Immune Escape and Fitness Constraints Compensated for by Intracodon Variation[▽]†

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The potential importance of HLA-C-restricted CD8$^+$ cytotoxic T lymphocytes (CTL) in HIV infection remains undetermined. We studied the dominant HLA-Cw*03-restricted CTL response to YVDRFFKTL$_{296-304}$ (YL9), within the conserved major homology region (MHR) of the Gag protein, in 80 HLA-Cw*03-positive individuals with chronic HIV infection to better define the efficacy of the YL9 HLA-C-restricted response. The HLA-Cw*03 allele is strongly associated with HIV sequence changes from Thr-303 to Val, Ile, or Ala at position 8 within the YL9 epitope ($P = 1.62 \times 10^{-10}$). *In vitro* studies revealed that introduction of the changes T303I and T303A into the YL9 epitope both significantly reduced CTL recognition and substantially reduced the viral replicative capacity. However, subsequent selection of the Val-303 variant, via intracodon variation from Ile-303 (I303V) or Ala-303 (A303V), restored both viral fitness and CTL recognition, as supported by our *in vivo* data. These results illustrate that HLA-C-restricted CTL responses are capable of driving viral immune escape within Gag, but in contrast to what was previously described for HLA-B-restricted Gag escape mutants, the common Cw*03-Gag-303V variant selected resulted in no detectable benefit to the host.

Human leukocyte antigen (HLA) class I is the most polymorphic region of the human genome. HLA class I genes are found at the A, B, and C loci of chromosome 6 and have been shown to play an important role in control of infections by intracellular pathogens (3). Of these three loci, HLA-B has had many more unique molecules identified than has HLA-A or -C (http://www.anthonynolan.org.uk/HIG/index.html). This locus variability is likely to reflect functional differences among HLA-B alleles and disease progression (20). In the context of HIV infection, several studies have demonstrated that HLA-B alleles have the greatest impact on HIV-1 replication control (7, 22). The reason why particular HLA-B, and not HLA-A or -C, alleles have been associated with improved outcome in HIV is unknown but may be explained by the numbers and regions of viral proteomes presented in the context of HLA-B molecules (19, 23, 46).

Regardless of the importance of HLA-B-restricted responses in HIV control, the roles of the HLA-A and -C class I alleles have not been fully investigated. A genomewide analysis investigating single-nucleotide polymorphisms (SNP) has associated both the presence of certain HLA-B alleles and a dimorphism upstream of the HLA-C gene (−35C/T) as two of the strongest predictors of the plasma viral load in HIV infection (14). In addition, the −35 SNP has recently been associated with levels of HLA-C expression and may indicate a new role for HLA-C alleles in HIV-1 control (40).

One reason that has been postulated to explain the lack of HLA-C association with immune pressure is the lower expression of HLA-C on the cell surface (18, 36, 37). However, HLA-C, unlike HLA-A and HLA-B, is not downregulated by the Nef protein, and this factor, therefore, may explain the lower expression level (9).

Analyses of large numbers of HIV sequences have identified associations between particular HLA-C polymorphisms in the HIV *pol* gene and the set point viral load (27). These polymorphisms were within or in close proximity to defined HLA-C-restricted epitopes, suggesting that HLA-C-restricted responses could be driving HIV evolution (22, 27). Confirmation that all HLA-C-restricted epitopes are not fundamentally ineffective was recently provided by a study of an HLA-Cw*01-restricted epitope targeted to p15 Gag, which was found to

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drive selection of a 3-amino-acid insertion in a HIV-infected patient (6). Additionally, *in vitro* studies have demonstrated antiviral activities of HLA-C-restricted cytotoxic T lymphocytes (CTL) clones against HIV comparable to other HLA-B-restricted responses (2). In spite of all these data, the contribution of HLA-C responses to HIV control is still unknown.

Our study was undertaken to address the role of an HLA-Cw*0303/0304-restricted response to $\text{YVDRFFKTL}_{296\text{-}304}$ (YL9) in HIV infection. This response was of particular interest due to its dominance and because it targets a highly conserved region of the Gag protein among different retroviruses, namely, the major homology region (MHR). In the study cohort of 778 individuals with chronic HIV infection, 80 were found to carry Cw*0303/0304. Gag viral sequences were obtained from HLA-Cw*0303/0304 subjects and used to identify and define HIV immune escape driven by the YL9-specific responses. We then studied the impacts of these mutations on viral recognition by $CD8^+$ T cells and on virus replicative capacity (RC).

MATERIALS AND METHODS

Study subjects. A total cohort of 778 subjects infected with C-clade HIV was studied. Of these, 578 individuals were recruited in Kwa-Zulu Natal (KZN) Province in South Africa, and 200 C-clade-infected subjects originally from sub-Saharan Africa were recruited in Oxford, United Kingdom, including the mother-child pair SL17. All individuals were naïve to antiretroviral therapy, and clinical data ($CD4^+$ count and viral load) were available. However, information regarding the time of infection was not available, as all patients were chronically infected by the time of analysis.

ELISPOT screening. A total of 778 study subjects were screened for HIV immune responses. Comprehensive epitope screening was achieved by incubating peripheral blood mononuclear cells (PBMCs) with overlapping peptides (OLPs) spanning the entire HIV proteome and measuring the subsequent gamma interferon (IFN-γ) response in an enzyme-linked immunospot (ELISPOT) assay as previously described (1). Of the 778 subjects, 80 were identified as carrying either HLA-Cw*0303 or Cw*0304 by high-resolution HLA typing (5). The HIV *gag* gene was sequenced from DNA from 640 C-clade-infected individuals. Sequencing was performed with primers published and described previously (24). Of the 80 subjects with HLA-Cw*0303/0304 and ELISPOT data available, 62 also had HIV Gag sequences.

YL9 epitope and variant recognition experiments. The YL9 epitope had previously been defined and shown to be restricted by the HLA-Cw*0303 and -0304 alleles (22). Peptides representing the amino acid changes T303A/I/V were synthesized (purity, 80%) (MBS 396; Advanced Chemtech, Louisville, KY; MGH Peptide Core Facility automated synthesizer). Recognition of the variant peptides compared to the wild type was determined by measurement of the IFN-γ response to serially diluted peptides for each variant in an ELISPOT assay. This was performed using PBMCs isolated from individuals ($n = 7$) responding to the wild-type YL9, as previously described (15).

Site-directed mutagenesis. Mutations in the HIV Gag protein at positions T303A, T303V, and T303I were introduced by site-directed mutagenesis in the *gag* gene into the p83-2 plasmid (Quikchange I; Stratagene, United Kingdom). The whole plasmid DNA was PCR amplified in a mutagenesis reaction with two complementary primers containing the target mutation. The primers used for the mutagenesis reaction were as follows: T303A, 5′-GAGACTATGTAGACCGATTCTATAAAGCTCTAAGAGCCGAG-3′ and 3′-CTCGGCTCTTAGAGCTTTATAGAATCGGTCTACATAGTCTC-5′; T303I, 5′-GAGACTATGTAGACCGATATAAAATTCTAAGAGCCGAGC-3′ and 3′-GCTCGGCTCTTAGAATTTTATAGAATCGGTCTACATAGTCTC-5′; and T303V, 5′-GAGACTATGTAGACCGATTCTATAAAGTTCTAAGAGCCGAG-3′ and 3′-CTCGGCTCTTAGAACTTTATAGAATCGGTCTACATAGTCTC-5′. The introduction of each mutation was verified by sequencing the whole *gag* gene in newly generated plasmid clones. The DNA fragment ranging from SapI to ApaI was then subcloned into a new p83-2 vector to avoid potential carryover of additional mutations during the mutagenesis, and the coding region sequence was verified again as previously described (26).

Virus production. Viral stocks were produced by cotransfection in MT4 cells of p83-2 site-directed mutants and p83-10eGFP as previously described (44). Viral stocks were harvested, and viral RNA was extracted (Qiagen, United Kingdom). The *gag* and *pol* genes were PCR amplified and sequenced to confirm the presence of the introduced mutations and the absence of other potential variations. The 50% tissue culture infective dose ($TCID_{50}$) for each viral stock was determined in MT4 cells using the method of Reed and Muench (33a).

Replication kinetics in Jurkat T cells and primary cells. Jurkat T cells were infected in triplicate to a multiplicity of infection (MOI) of 0.001 in a total volume of 1 ml with wild-type virus or virus variants and incubated at 37°C for 2 h. The pellets were washed twice with phosphate-buffered saline (PBS) and cultured at 37°C and 5% CO_2, as previously described (31). Viral infectivity was determined daily for 15 days using fluorescence-activated cell sorting (FACS) analysis to measure the percentage of enhanced green fluorescent protein (EGFP)-positive cells, as previously described (32). A similar experiment was performed in peripheral blood mononuclear cells (PBMCs) by purifying these cells from a seronegative donor and stimulating them with phytohemagglutinin (PHA) at 5 μg/ml and 0.5 μg/ml for 72 h. After stimulation, the cells were pulled together, and the cellular pellets were infected with each viral variant at an MOI of 0.02 for 2 h at 37°C. The cellular pellets were washed twice with PBS and cultured at 37°C and 5% CO_2. PBMCs were resuspended in R10 medium supplemented with interleukin 2 (IL-2) (Roche, United Kingdom) at 100 U/ml to a final concentration of 1×10^6 cells/ml. Viral spread and infectivity were measured as the percentage of CD4-p24-positive cells by intracellular staining with anti-CD3 and anti-CD4 antibodies (Becton Dickinson) and HIV Gag-p24 phycoerythrin (PE) clone KC57-RD1 (Coulter Clone, United Kingdom). This analysis was performed using FACS. The FACS data were analyzed with Flowjo v8.6.3.

Clonal and phylogenetic analyses. Detailed analysis of proviral DNA clones was undertaken for two HLA-Cw*0304-positive individuals within the Oxford cohort: the child, SL17C, of a mother-to-child transmission pair (SL17M/SL17C) and an individual (H004) with the T303V variant as a population sequence. The HIV *gag* gene was PCR amplified and cloned into the Topo vector (Invitrogen, United Kingdom). Approximately 20 to 30 clones were sequenced per time point and individual. The H004 and SL17M/SL17C sequences were edited and aligned with SeqAlv2.0. jModeltest v0.1.1 (30) was used to infer the best phylogenetic model that was able to implement a discrete gamma distribution (Γ), which models the heterogeneity rate among sites. A maximum-likelihood tree was obtained with PhyML v3.0 (17), with the best evolutionary model found in jModeltest. In order to assess the significance of the nodes, 1,000 bootstrap replicates were performed, and only nodes with bootstrap values higher that 70% were taken to be significant. The phylogenetic trees were edited using FigTree v1.1.2 (http://tree.bio.ed.ac.uk/).

Statistical analysis. A 2-tailed Fisher's exact test was used to identify the associations between alleles and specific polymorphisms. PRISM software was used for the analysis of viral kinetics, CD4, and viral-load data sets. The infectivity rate of each virus was analyzed by fitting a linear model to the $\log_{10}$-transformed data of EGFP expression and comparing the slopes, as previously described (32). The slopes were considered significantly different if P was <0.05. The Mann-Whitney test was used to compare viral loads between groups, and a 2-tailed unpaired t test was use to compare absolute CD4 counts.

RESULTS

Chronically HIV-infected HLA-Cw*0303/0304-positive individuals make an immunodominant Gag-296-304-specific response. Comprehensive screening using a panel of 410 OLPs spanning the whole HIV proteome showed a high frequency of responses in HLA-Cw*0303/0304-positive subjects against the adjacent Gag peptides OLP-40 (GPKEPFRDYVRFFKTLR) and OLP-41 (YVDRFFKTLRAEQATQDV), with 70 of 80 (87.5%) chronically infected individuals having a detectable response (Fig. 1A). These OLPs contain the HLA-Cw*0303/0304 optimal restricted epitope YL9, which was previously defined (22). The magnitude of these responses made up >50% of the total Gag responses in almost one-third of Cw*03-positive individuals (26/80) and 100% in 15% of these individuals (Fig. 1B) (this calculation counted responses to OLP-40 or OLP-41, whichever was greater, in order to avoid counting the YL9 response twice). In addition, previous, smaller studies on 24 Cw*0304-positive subjects of the same

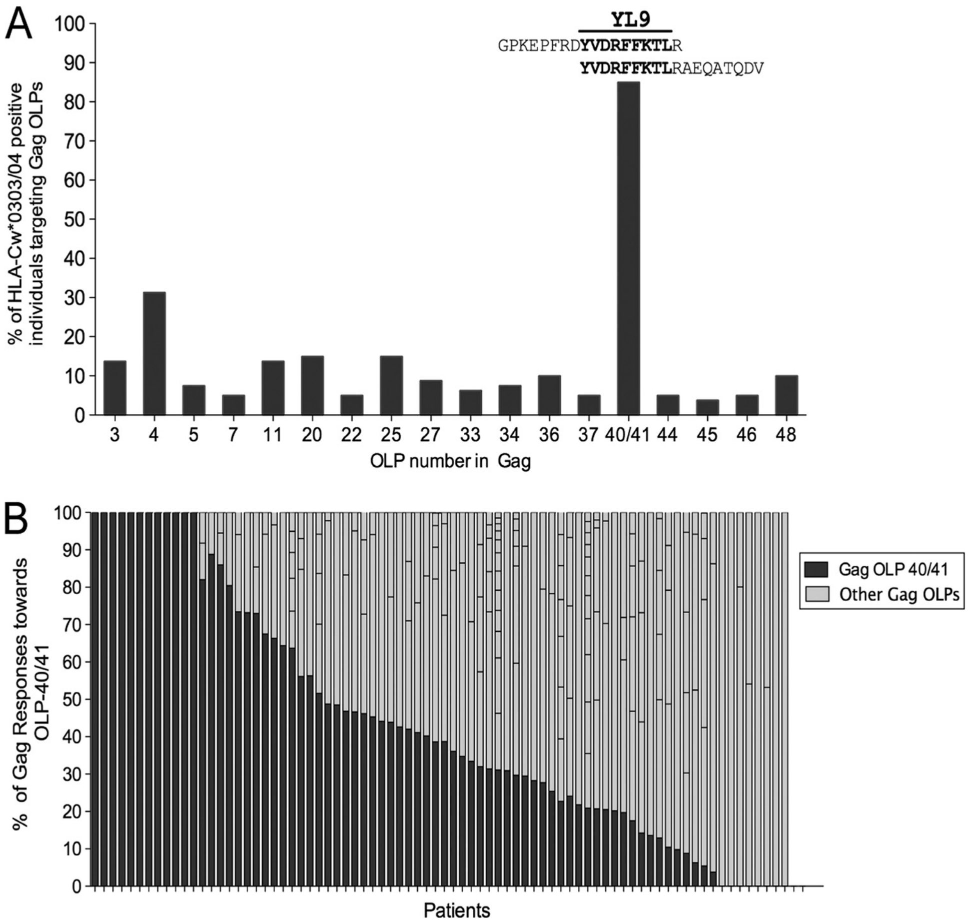


FIG. 1. (A) Percentages of HLA-Cw*0303/0304 chronically HIV-infected individuals (n = 80) with IFN-γ responses against a panel of HIV Gag OLPs. Above the bars are shown the amino acid sequences for OLP-40 and -41 containing the optimal epitope YL9, represented in boldface. Only those responses observed at a frequency higher than 5% are shown in the graph. (B) Percentages of Gag responses to OLP-40/41 (black bars) related to the total magnitude of Gag responses (gray bars) in HLA-Cw*0303/0304-positive individuals.

cohort (12), using the Cw*0304-YL9 tetramer, showed detectable responses to this epitope, with a median response frequency of 2.26% of CD8$^+$ T cells, the highest of 8 immunodominant responses that were examined. Furthermore, although it is possible that responses to additional optimal epitopes may have contributed to the responses to the overlapping 18-mer peptides OLP-40/41, in 7 Cw*0304-positive subjects selected on the basis of cell availability for further study, all subjects having responses to OLP-40 or -41 also had a detectable response to the YL9 optimal restricted epitope. Additionally, the magnitude of the YL9 response correlated strongly with that of the response to the OLP and was statistically significant in spite of the small numbers (n = 7) of subjects evaluated (for example, for YL9 and OLP-41, r^2 was 0.67 and P was 0.02 [data not shown]). No other HLA-Cw*0303- or Cw*0304-restricted HIV-specific responses have been defined to date. Based on these data, the HLA-Cw*0303/04-restricted CTL responses directed to YL9 in Gag are immunodominant, both in terms of the frequency with which the epitope is targeted in Cw*03-positive individuals and also in the magnitude of the YL9-specific response compared to responses to other epitopes in Gag.

HLA-Cw*0303/0304 selects variants in Gag-303 that reduce YL9 immune recognition. In order to determine whether the HLA-Cw*0303/0304-YL9 response imposes selection pressure on the virus, we analyzed RNA and DNA *gag* sequences from 640 clade C-infected study subjects. In the region of the YL9 epitope, we identified a strong association between HLA-Cw*0303/0304 and the polymorphisms Val/Ile/Ala/Cys at Gag residue 303 ($P = 1.62 \times 10^{-10}$) (Table 1). Viruses from 45% (28/62) of the subjects carried one of these changes compared to 11% (61/578) of Cw*0303/0304-negative subjects. The most common variants in Cw*03-positive subjects in place of wild-type Thr-303 were valine (39% of variants), alanine (29%), and isoleucine (25%), with cysteine (7%) the least common (T303C was not studied in further detail, since it was very rare in the Cw*0303/0304 population) (Table 2).

To examine the impact of the Cw*0303/04-associated Gag-303 variants on YL9 recognition, synthetic YL9 peptides containing the T303V, T303A, and T303I mutations were synthesized, and their recognition by CD8$^+$ T cells was compared to that of the wild-type epitope in an IFN-γ ELISPOT assay of PBMCs from seven Cw*0303/04 individuals based on sample availability (1). The T303A and T303I mutants were less well

TABLE 1. Variation in the consensus sequence of the epitope Cw*0303/0304-YVDRFFKTL, Gag residues 296 to 304, and 5 amino acids beyond the N and C termini for Cw*0303/0304-positive and Cw*0303/0304-negative individuals

WT amino acid	HXB2a reference	Mutation	No. of sequences				*P* value (Fisher's exact test)[a]
			Cw*0303/0304$^+$		Cw*0303/0304$^-$		
			No mutation	Mutation	No mutation	Mutation	
291	E	X	62	0	578	0	NS
292	P	ST	62	0	569	9	NS
293	F	X	62	0	578	0	NS
294	R	X	62	0	578	0	NS
295	D	E	60	2	574	4	NS
296	**Y**	X	61	1	577	1	NS
297	**V**	X	62	0	578	0	NS
298	**D**	X	61	1	576	2	NS
299	**R**	X	62	0	578	0	NS
300	**F**	X	62	0	578	0	NS
301	**F**	Y	62	0	574	4	NS
302	**K**	R	61	1	563	14	NS
303	**T**	**VIAC**	34	28	517	61	**1.62×10^{-10}**
304	**L**	X	62	0	578	0	NS
305	R	X	62	0	576	2	NS
306	A	X	62	0	574	4	NS
307	E	D	62	0	577	1	NS
308	Q	X	62	0	578	0	NS
309	A	GS	57	5	545	33	NS

[a] NS, not significant.

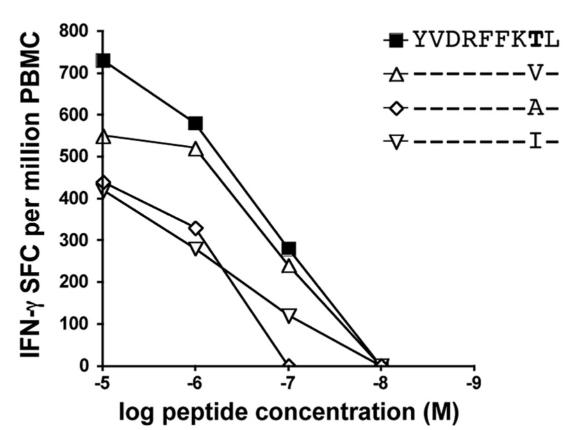
recognized than the wild-type YL9 epitope (median, 7 study subjects; 50% saturating doses (SD_{50}) , 243 nM, 235 nM, and 47 nM, respectively) (Fig. 2B), but only a marginal effect on the SD_{50} was found with the individually more common T303V mutation (median SD_{50}, 75 nM) (Fig. 2A). These data suggest that immune-mediated selective pressure on HIV is driven by HLA-Cw*0303/0304 through selection of T303V/A/I/C variants at position 8 of the YL9 epitope. The escape variants T303V/A/I at position 303 reduce, but do not abrogate, YL9 recognition.

***In vitro* viral replicative capacities of common YL9 T303 variants.** It is known that HIV immune escape is limited by structural constraints (16). In addition, particular CTL escape mutations in HIV Gag-p24 have been associated with a reduction in the viral RC (4, 11, 26, 32). Structurally, the YL9 Gag responses target the MHR of the Gag p24 protein, a region of the HIV proteome that is highly conserved between retroviruses (25). The MHR is located within helix 8 of the C-terminal domain of p24 (see Fig. S1 in the supplemental material) and has been implicated in virus particle assembly (8). To examine the effect on virus RC of HIV escape variants selected by an HLA-C-restricted response, mutations at residues T303V/I/A in YL9 were introduced into the p83-2 vector

TABLE 2. Prevalences of variants at residue 303 for individuals with Cw*0303/0304 and for those not carrying the allele

Residue at 303	No. (%) of sequences	
	Cw*0303/0304$^+$	Cw*0303/0304$^-$
T	34 (54.8)	517 (89.4)
V	11 (17.7)	41 (7.1)
A	8 (13.0)	4 (0.7)
I	7 (11.3)	4 (0.7)
C	2 (3.2)	12 (2.1)

A



B

Subject	SD_{50} (nM)				Population Sequence
	YVDRFF**T**L	YVDRFF**V**L	YVDRFF**I**L	YVDRFF**A**L	YVDRFF**T**L
H020	94	95	332	342	---------
H018	133	95	591	97	---------
N019	30	38	59	61	---------
N013	12	134	235	243	---------
R027	24	30	118	77	-------A-
H004	237	238	373	768	-------V-
H016	47	24	74	967	-------C-
Median	**47**	**95**	**236**	**243**	

FIG. 2. (A) Recognition of YL9 variant peptides serially diluted and measured in an IFN-γ ELISPOT assay in PBMCs from a Cw*0304-positive individual. (B) Variant recognition in PBMCs from HLA-Cw*0303/0304 chronically HIV-infected individuals (n = 7). SFC, spot-forming cells.

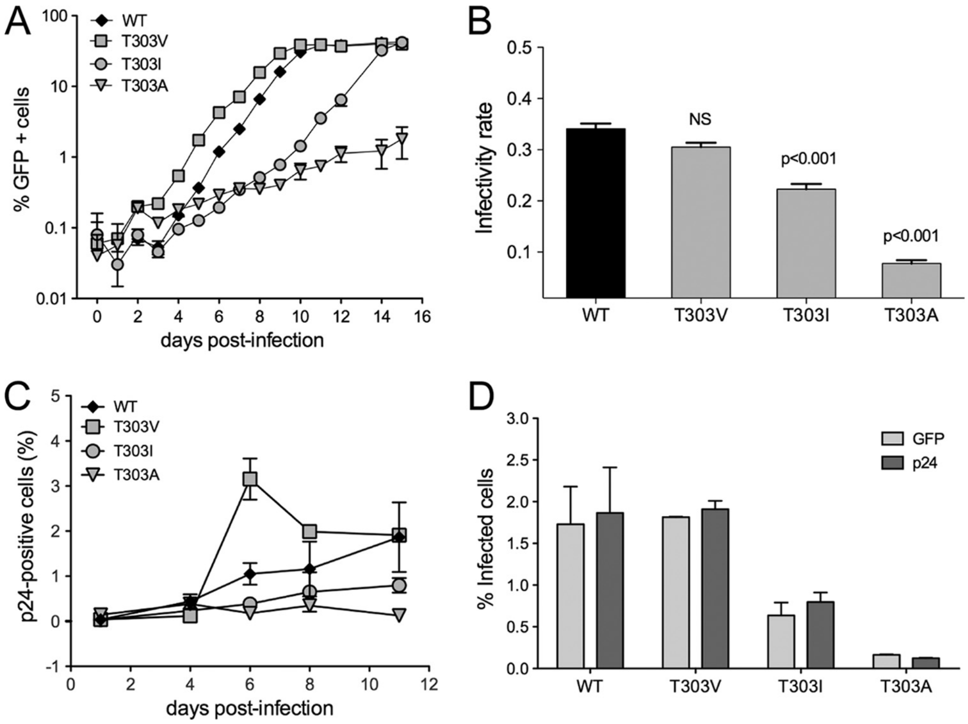


FIG. 3. Replication kinetics of YL9 mutants. (A) Jurkat T cells were infected in triplicate at an MOI of 0.001. Viral infection was measured over time by FACS analysis of the percentage of GFP^+ cells. WT, wild type. The error bars represent standard deviations. (B) Infectivity rates based on the slopes of the different lines from panel A were compared by linear regression (a *P* value of <0.01 was considered significant; NS denotes nonsignificant data). The bars represent means and standard deviations. (C) Replication kinetics in PBMCs. PBMCs were infected in triplicate with viral variants at an MOI of 0.02. (D) Comparison between the percentages of GFP^+ cells and intracellular $p24^+$ cells at day 11 after infection. The bars represent means and standard deviations.

(NL43 backbone), and the virus RC was measured in Jurkat T-cell and primary cultures, as previously described (32). Viruses with the YL9 escape mutations T303A and T303I were demonstrated to have substantially decreased infectivity rates and impaired kinetics compared to wild-type virus (Fig. 3A and B). Furthermore, introduction of T303V was found to increase the virus RC to levels similar to that of wild-type virus. Comparable results were obtained in replication kinetics experiments in PBMCs measured by both GFP^+ and intracellular p24 staining (Fig. 3C and D). In addition, all viruses were sequenced at the end of the replication kinetics experiments. No reversion to wild type or additional compensatory changes were observed in the Gag coding region for any of the T303 variants. Overall, these results indeed demonstrate that Cw*03-restricted YL9 responses induce changes in HIV RC through viral immune escape. However, the most commonly observed variant, T303V, resulted in minimal loss of $CD8^+$ T-cell recognition, with no detectable reduction in the viral RC.

Balance between YL9 immune escape and viral replicative capacity drives two-step nucleotide substitution concomitant with a decrease in absolute CD4 counts and an increase in the viral load. T303V is the most common variant in the population. However, T303V requires 2 nucleotide substitutions from the T303 wild type compared to the single substitution needed for T303A or T303I selection (Fig. 4A). Therefore, selection of T303V involves the previous selection of the immune escape variant T303A or T303I. To examine in more detail the selection of these distinct YL9 escape mutants, the *gag* gene was PCR amplified from a Cw*0304-positive infected individual for whom T303V had previously been determined to be the predominant (population) sequence, and proviral DNA clonal analyses were carried out. Phylogenetic analysis showed the coexistence of viral populations encoding the T303A/V viral variants, together with the wild type, in the same subject (Fig. 4B). This result suggests the presence of the intermediate form, T303A, en route to T303V, but not the outgrowth of T303A in the viral population.

These data suggest that, for most subjects with Cw*0303/04, a balance exists between the selection of the T303I/A variants poorly recognized by CTL but significantly reducing viral replicative capacity and the selection of T303V, where viral escape is modest but the reduction in viral replicative capacity is also minimal. Comparison of absolute CD4 counts and viral loads in Cw*03-positive individuals (Fig. 4C and D) suggested that selection of T303V brings about the optimal result for the virus: Meanwhile the T303I/A mutations overall do not have substantial impacts on the viral load or CD4 counts (median viral load, 19,900 RNA copies/ml; median CD4 count, 373 cells/μl) compared with those individuals with wild-type virus (median viral load, 25,900 copies/ml; median CD4 count, 433 cells/μl); Cw*03-positive subjects carrying the T303V mutants had a median viral load of 96,550 HIV RNA copies/ml, almost 5-fold higher than those individuals who had virus with the wild-type or T303I/A mutants (P = 0.096; Mann-Whitney U test) and half of the absolute CD4 numbers (203 cells/μl; P =

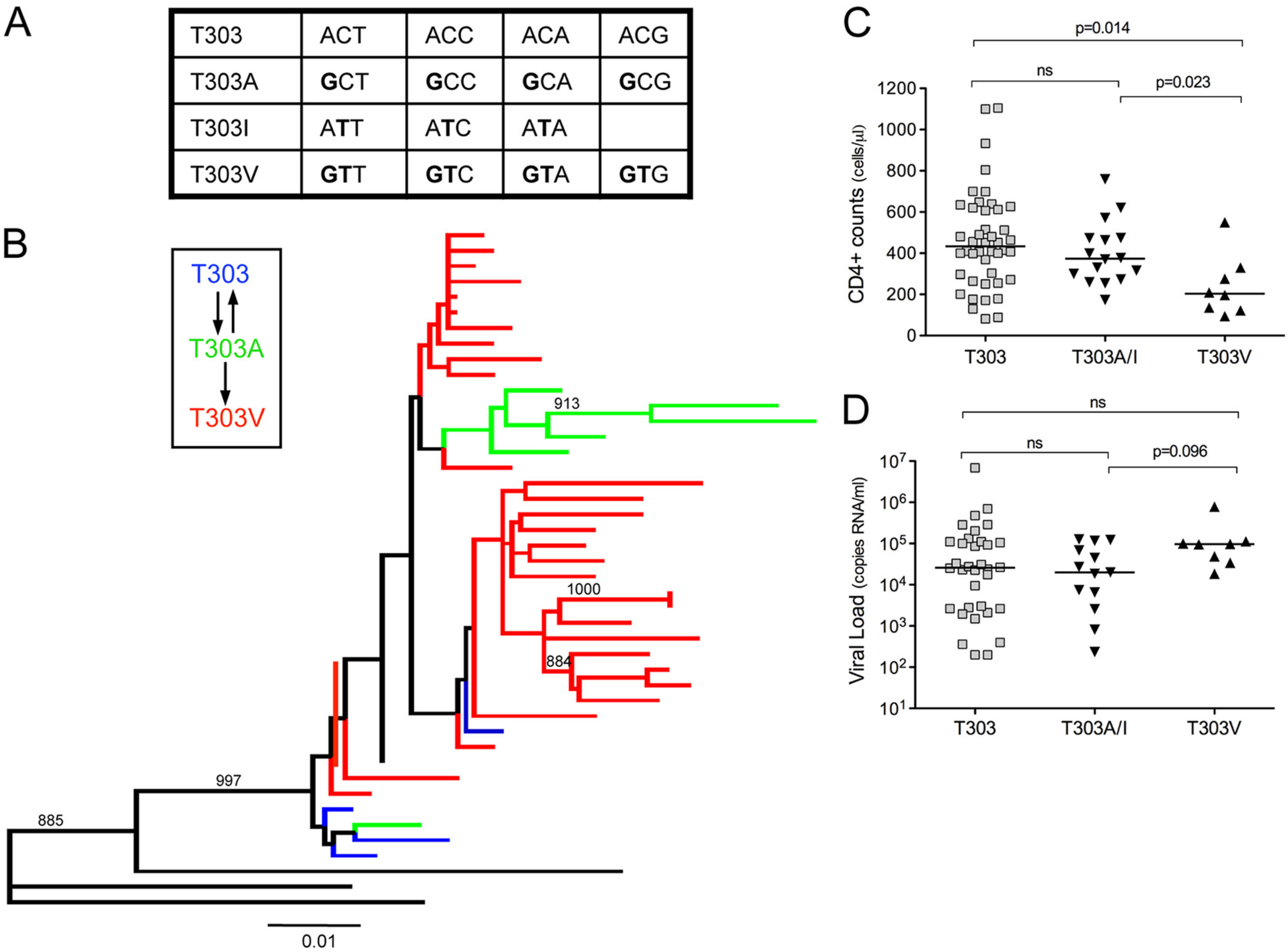


FIG. 4. (A) Nucleotide usage at codon 303. The table shows the nucleotide usage required for the appearance of mutations at YL9 from wild-type T303 to T303I/A and T303V. Nucleotide substitutions required for an amino acid change are represented in boldface. (B) Maximum-likelihood phylogenetic tree for subject H004 using the HKY + I + Γ model, where Γ = gamma distribution with an alpha values of 0.75. Each variant is represented by a different color (T303V, red; T303A, green; T303, blue). The boxed chart shows the potential pathway for 303V selection. The black lines represent population sequences from other patients used as an outgroup for the tree. The node numbers represent bootstrap values with scores higher than 70%. (C and D) Median CD4 counts and median viral loads of HLA-Cw*03 individuals. CD4 and viral loads were grouped for those individuals who had virus containing wild-type sequence (T303), T303A/I, and T303V (The *P* value for the viral load was derived using a Mann-Whitney U test, and a *P* value of <0.05 was considered significant; the *P* value for CD4 counts was derived using 2-tailed unpaired *t* test, and a *P* value of <0.05 was considered significant).

0.023; 2-tailed unpaired *t* test). There was also a statistically significantly higher absolute CD4 count in the subjects whose autologous virus expressed the T303I/A variants than in those with T303V (absolute CD4 counts, 373/µl versus 203/µl; $P = 0.023$; 2-tailed unpaired *t* test) (Fig. 4C). These data suggest either that Gag-303-V gives the virus the optimal result, increasing *in vivo* replication with unaffected virus RC and adequately reduced CTL recognition, or that the Gag-303-V variant may be selected in the setting of high viremia and a weak immune response against the virus at a time when selection pressure is not strong enough to select for escape mutants that reduce viral replicative capacity.

These findings are supported by data from an HIV-infected mother-child pair in which the expression of HLA-Cw*0304 in the infant selected the Val at position 303 in an 11-month study period (Fig. 5A). A maximum-likelihood phylogenetic tree for the mother-child pair demonstrated *in vivo* virus evolution toward T303V in the absence of minority variants, as opposed to the presence of T303A or T303I selected by the continuous and stronger immune pressure present in adults (Fig. 5B).

DISCUSSION

To date, few studies have investigated the role of HLA-C-restricted responses in HIV infection, and there are very limited data regarding the characterization of HLA-C-mediated immune responses. Previous studies have suggested that HLA-C-restricted CD8$^+$ T-cell responses are ineffective (23) and yet that HLA-C expression may be an important influence on HIV disease outcome (14). This work examined in detail the role of a Gag-specific HLA-Cw*03-restricted response with respect to HIV evolution at the population and individual levels. This was

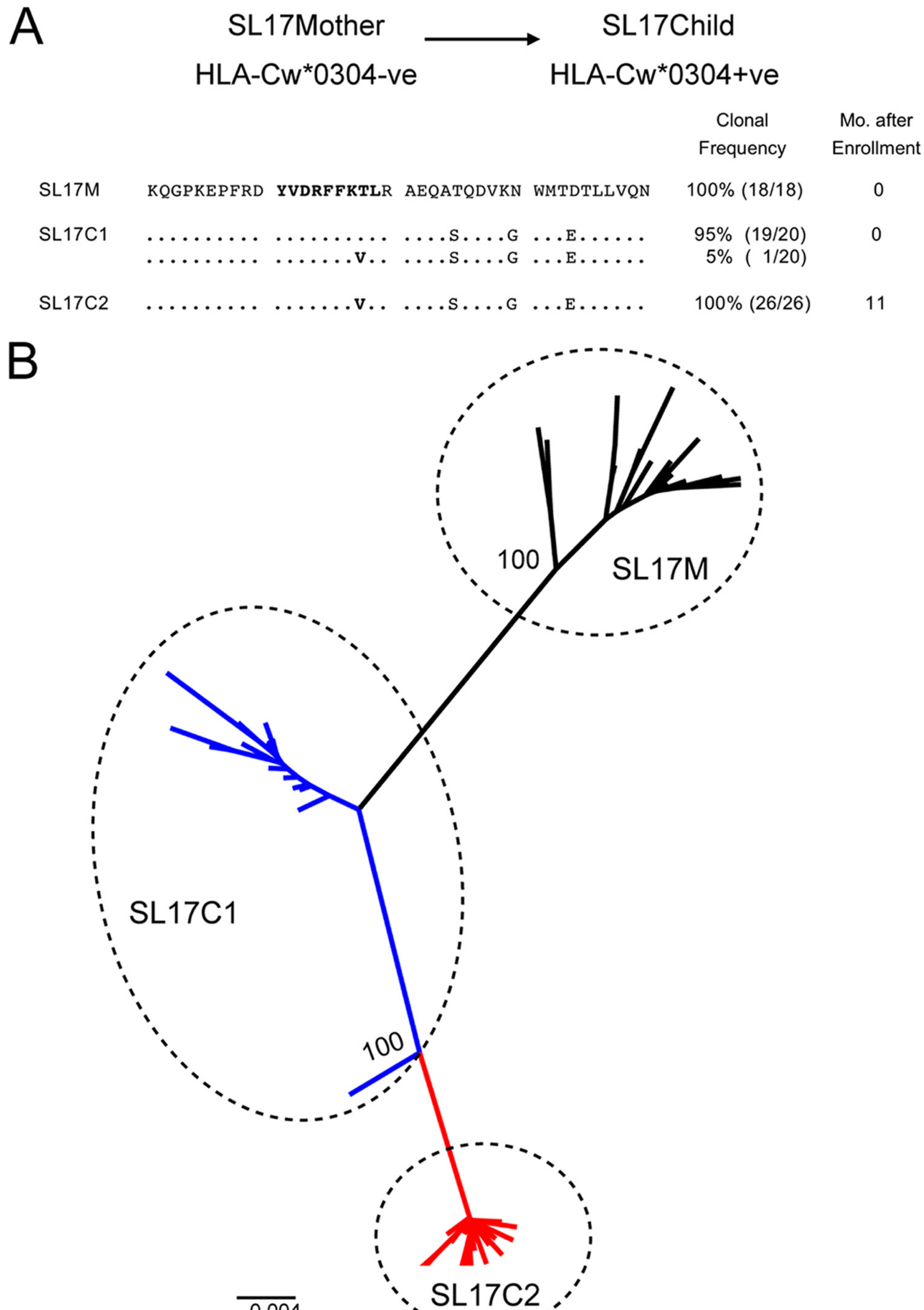


FIG. 5. (A) Clonal analyses of the SL17 mother-to-child transmission pairs. SL17M stands for the mother; SL17C stands for the children. The HLA type for SL17M is HLA-A*0201G/3303, HLA-B*4501/5801, Cw*0302/1601B, and the SL17C type is HLA-A*0201G/2601, HLA-B*1510/4501, HLA-Cw*0304/1601. Clonal frequency is expressed as a percentage for each variant based on the number of clones analyzed (shown in parentheses). Mo., months. The positions of the YL9 epitopes are shaded. (B) Maximum-likelihood phylogenetic tree based on a Tamura-Nei model with a Γ distribution ($\alpha = 0.664$) representing the SL17M/C pair. The circles indicate clusters of sequences. The black lines represent maternal virus sequences, while the blue and red lines represent infant sequences at time points 1 and 2, respectively. The node numbers represent bootstrap values with scores higher than 70%.

investigated by assessing the impact of Cw*03-YL9-specific CD8$^+$ T-cell responses on virus immune escape and fitness.

This study found that individuals with chronic HIV infection expressing the HLA-Cw*03 allele have an immunodominant CTL response against the Gag YL9 (YVDRFFKTL$_{296\text{-}304}$) epitope. YL9 responses drive virus sequence variation at position 8 of the YL9 epitope from Thr to Ala, Ile, or Val. Selection of these variants reduces epitope recognition for

T303A and T303I variants, but only marginally in the case of the Val substitution, by conventional *in vitro* peptide titration experiments. Although a more accurate description of the binding for each particular variant could be obtained by alternative methodologies, such as a CTL-killing assay, this was far from the purpose of this study.

HLA-Cw alleles have been less well studied than their HLA-A and HLA-B counterparts. The peptide-binding motif for HLA-Cw*0304 has been formally determined (13, 41, 45) and does not differ from the majority of other HLA class I alleles (38). HLA-Cw*0304 binds peptides with a small hydrophobic residue at P2 (such as alanine or valine) and a medium-size hydrophobic residue at PC (such as leucine). The HLA-Cw alleles for which the peptide-binding motif has been formally determined, via elution and pool sequencing of peptides from purified class I molecules, include, in addition to Cw*0304, HLA-Cw*0102 and Cw*0401, which are broadly similar in peptide-binding preference to Cw*0303, and Cw*0602 and Cw*0702, which preferentially bind peptides carrying arginine at P2.

The HLA-Cw*0303/0304-YL9 (Y**V**DRFFKTL) epitope thus corresponds to the motif defined for HLA-Cw*0304. Although the anchor residues are located at P2 and PC, other residues in the peptide may contribute to HLA binding, in particular residue 1 (binding into the A pocket), residue 3 (binding into the D pocket), and residues 5 and 6 (binding into the C pocket) (38). Changes at residue 8 (T303 in Cw*03-YL9) might therefore have some impact on HLA binding, but the principal impact of a P8 change would be expected to be on the T cell receptor interaction. However, additional effects not investigated in this study, in particular via altered processing, might also arise as a result of these mutations at Gag-303.

It is noteworthy that the preferential selection was observed in Cw*03-positive individuals with the T303V variant (39%), in spite of such a marginal effect in virus immune escape and with the extra requirement of two-step nucleotide substitution for selection. In order to further understand this phenomenon, we carried out *in vitro* experiments with viral RC. RC studies demonstrated the fitness advantage of T303V over the lower-replication T303I and -A variants, with T303V having an RC profile similar to that of the wild-type virus. A previous independent study showed reduction similar to what we have observed for the T303A variant in the context of an HLA-B*14 Gag-CD8^{+}-restricted response from a long-term nonprogressor (43).

The reason behind such a decrease in RC for Ala-303 and Ile-303 could be related to the position of the epitope within the Gag protein. The YL9 epitope is located in the MHR of the p24 protein, a region of the HIV proteome that is highly conserved between retroviruses (25). Notably, certain residues in the MHR are more conserved among retroviruses than others (10), and different amino acids appear to have varying biological effects during viral assembly. As previously reported, complete removal of the 20-amino-acid MHR section dramatically affected the ability of Gag to bind to the cell membrane and assemble virus-like particles (33). In addition, site-directed mutagenesis studies demonstrated that changes to Ala at position T303 resulted in a defect in Gag assembly to the cell membrane and suggest a protein-protein interaction deficiency for the T303A and T303I low-fitness variants (8). Structurally, one may speculate that the consequences of the T303V substitution might be less than those of either of the T303A/I substitutions simply because Val (molecular weight [MW], 117) is closer in size to Thr (MW, 119) than Ala or Ile (MW, 89 and 131, respectively), and thus would have less impact on optimal packing of the C-terminal domain of the viral capsid. The side chain hydroxyl group of Thr offers hydrogen-binding opportunities that would be expected to increase intra- and intermolecular capsid stability and that might potentially affect the orientation of the N-terminal and C-terminal capsid domains, given its location adjacent to the interdomain hinge region (42) (see Fig. S2 in the supplemental material). However, none of these T303A/V/I substitutions appears sufficiently disruptive to capsid structure to require the selection of compensatory mutants to restore structural stability: First, our longitudinal analysis from clonal sequences obtained from the child SL17, in whom T303V was selected, did not show additional putative compensatory mutants coincident with or subsequent to T303V selection. Second, our cross-sectional analyses of the cohort overall did not show any association between the variation at position 303 and selection at other positions in Gag, supporting the lack of compensatory changes resulting from the selection of T303V. Third, the use of site-directed mutants and the sequencing data at the end of the replication experiments did not reveal the acquisition of additional mutations during the *in vitro* culture. These results support the full replicative capacity of the T303V variants without variation at other sites of the Gag protein.

Our data provide the first direct evidence that selection of escape mutations in conserved regions of the virus by CTL directed to HLA-C-restricted epitopes can result in fitness constraints similar to those previously described for other CTLs directed to epitopes restricted by HLA-B (11, 26). However, in contrast to what has been observed in relation to certain HLA-B-associated Gag mutants (27), the most frequently selected HLA-Cw*03-driven variant, T303V, ultimately resulted in no fitness cost, concomitant with a substantial increase in the viral load and a decrease in the CD4 count for those Cw*03-positive subjects carrying the T303V variant. These data suggest that the absolute CD4 count declines faster in subjects with T303V or that T303V is observed in subjects who have been infected for a longer period. In this cohort, the duration of infection is unknown, and therefore, these two possibilities cannot be distinguished. However, these data are consistent with T303V representing the optimal solution for the virus, i.e., CD8^{+} T-cell escape at minimal cost to viral replicative capacity.

HIV has enormous flexibility to overcome immune pressure exerted by CTLs that results in fitness constraints (21, 26). To date, studies by our group and others have described mechanisms that compensate for the fitness costs associated with virus immune escape in the Gag protein. Compensatory mutations have been demonstrated to occur within the same epitope (11) or outside the epitope but nearby in the three-dimensional protein structure (4, 34). Here, we have documented a novel compensatory mechanism via intracodon substitution and two-step nucleotide selection of the T303V through T303A or -I intermediates. These data provide a further example of the plasticity of HIV to defeat the structural constraints imposed by the immune system.

The studies described here illustrate the balance between viral fitness and selection of immune escape variants in the context of minimal recognition. At the individual level, phylogenetic analyses demonstrated the presence of T303V in chronic HIV infection in spite of the selection of a minority of low-fitness intermediate viral variants. These data contrast with the lack of detection of those intermediates in the infected child in whom YL9 responses drove virus evolution rapidly toward T303V. Although this is a single anecdotal case, it is possible that the presence of T303A/I intermediates in adults, or in subjects with lower viral loads, could be explained by a more effective $CD8^+$ T-cell response capable of driving sufficiently strong selection for variants that are poorly seen by the CTLs (i.e., T303A and T303I), even though they reduce the viral RC. Therefore, T303A and -I mutants will be selected as a pool of minor low-replication variants that will rapidly disappear in favor of the T303V variants. Also, these data suggest that Gag-303-V is selected in the setting of a weak immune response and consistent with the narrower immune response that is typically observed in pediatric infection or in adults with more advanced disease (39). Escape has also been described previously in the child, but not the mother, of a mother-child pair where the HLA alleles and the response were shared (29). Our results illustrate the fine balance that exists between opposing selection forces and the fact that this balance may shift during the course of infection within a single individual as disease progresses, as well as differing between individuals.

In summary, the HLA-Cw*03-YL9-restricted response preferentially selects the fittest and best-recognized viral variants as the major circulating species. The data presented document the ability of certain HLA-C-restricted $CD8^+$ T-cell responses to mediate immune pressure on HIV-1, driving virus escape and fitness constraints. However, further studies of additional HLA-C-restricted responses will help to clarify whether the Cw*03-restricted Gag response analyzed here is representative of HLA-C-restricted $CD8^+$ T-cell responses.

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